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Expression of CD34 on human B cell precursors

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SUMMARY

CD34 is a 110-kD glycoprotein previously shown by a variety of monoclonal antibodies (MoAbs) to be expressed selectively on immature hematopoietic cells. However, more detailed characterization of $CD34^+$ cells has been hampered by lack of anti-CD34 MoAbs that can be labelled directly with fluorochromes to facilitate subpopulation analysis by multi-parameter flow cytometry. We have recently isolated a murine anti-CD34 MoAb, designated as 8G12, that can be directly labelled with fluorochromes such as FITC. In this study, we have exploited this property of 8G12 to compare the reactivity of 8G12 and My10 with normal and leukaemic human marrow cells and to characterize normal early human B cell precursors by two- and three-colour immunofluorescence analysis. Comparison of three-colour staining profiles of normal bone marrow cells incubated with both 8G12 and MY10, and either anti-CD10 or anti-CD19 MoAb revealed the reactivity patterns of 8G12 and MY10 to be indistinguishable. This conclusion was confirmed by a similar comparative analysis of 8G12 and MY10 staining of blood and bone marrow cells from 4 patients with B lineage acute lymphoblastic leukaemia (ALL). Of interest, both 8G12 and MY10 detected a $CD34^+CD10^+CD19^+$ population in normal adult bone marrow. To determine whether a $CD34^+CD10^+CD19^-$ precursor population previously reported by others to exist in fetal liver could also be identified, $CD10^+CD16^-$ marrow cells were first isolated by FACS and the sorted cells then re-analysed for expression of CD19 and CD34. These studies showed that all of the sorted $CD10^+$ cells that expressed CD34 appeared to coexpress CD19. No $CD34^+CD10^+CD19^-$ cells were detected (at a sensitivity of $\leq 0{\cdot}1\%$). Further studies will be required to determine whether a very minor population of $CD34^+CD10^+CD19^-$ cells may still be generated in the normal development of B cells in adult human marrow.

Keywords B cell precursors CD34 ontogeny FACS analysis

INTRODUCTION

CD34 is a human stage-specific hematopoietic differentiation antigen expressed on immature cells of multiple lineages including lymphoid and myeloid pathways (Civin *et al.*, 1984; Tindle *et al.*, 1985; Andrews, Singer & Bernstein, 1986; Civin *et al.*, 1987). About 1–4% of low-density bone marrow cells express readily detectable levels of CD34 antigen on the cell surface and this includes virtually all of the myelopoietic colony-forming cells. The most primitive haematopoietic progenitors show the highest CD34 expression which is then progressively lost with maturation (Lu *et al.*, 1987; Andrews, Singer & Bernstein, 1989; Sutherland *et al.*, 1989). Consistent with this are indications that transplantation of autologous populations enriched in $CD34^+$ cells may restore haematopoiesis in lethally irradiated baboons (Berenson *et al.*, 1988) or in cancer patients treated with myeloablative chemotherapy (Berenson *et al.*, 1989).

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As might be anticipated from the developmentally restricted expression of CD34 to early haematopoietic cells, a large percentage of acute leukaemias are $CD34^+$, including 60% of B-lineage acute lymphoid leukaemia (ALL), 40% of acute myeloid leukaemia (AML) and some T-lineage ALLs. Similarly, chronic lymphoid leukaemias and lymphomas, which represent malignancies originating from more mature cell types, are uniformly $CD34^-$ (Civin *et al.*, 1989). The stage-specific expression of CD34 has been particularly useful for investigating the early differentiation of normal human B-lineage cells. Since functional assays for these cells do not exist, most information to date has been derived from phenotype analyses. Studies of cells from some ALL patients have suggested that expression of CD19 (Leu12, B4) can occur in the absence of CD10 (CALLA) (Nadler *et al.*, 1984; Hokland *et al.*, 1985; Uckun & Ledbetter, 1988). However, controversy exists as to the sequence of CD10 and CD19 expression during normal B-lineage development (Loken *et al.*, 1987;Uckun & Ledbetter, 1988; Hurwitz *et al.*, 1988; Loken *et al.*, 1988; Smith & Kitchens 1989). Although the existence of an early stage of B cell precursors with the $CD34^+CD10^+CD19^-$ phenotype has been reported in fetal

liver (Uckun & Ledbetter, 1988), our results suggest concomitant acquisition of CD10 and CD19 expression on human adult bone marrow B lymphoid precursors.

Several monoclonal antibodies (MoAbs) against CD34, which is expressed at the surface as a monomeric glycoprotein of about 110 kD (Civin *et al.*, 1989), have been described. These antibodies include MY10, BI-3C5, 12.8, p115.2 and TUK3 and recognize at least two distinct CD34 epitopes (Lansdorp, Dougherty & Humphries, 1989). Although all strongly stain cells from the KG1a myeloblastic cell line, their reactivity with normal light density ($<1{\cdot}077$ g/cm^3) bone marrow cells is relatively weak due to their low affinity and the much lower CD34 antigen density on most normal haematopoietic progenitors (Civin *et al.*, 1987). A second problem encountered with the use of many of these anti-CD34 MoAbs for marrow studies is associated with their loss of reactivity after covalent coupling to fluorochromes which makes their use in multi-colour fluorescent analyses difficult. Attempts to directly label My10 with FITC or PE have been invariably unsuccessful (M. Loken, Mountain View, personal communication).

We now describe the staining characteristics of CD34+ subpopulations in normal marrow and in several ALL samples using a new IgG1 anti-CD34 MoAb (8G12) that does not compete with MY10 for binding to KG1a cells (Lansdorp *et al.*, 1989) and that can also be directly labelled with fluorochromes (Lansdorp, Sutherland & Eaves, 1990).

MATERIALS AND METHODS

Cells

Normal human marrow aspirate samples were obtained with informed consent from individuals donating marrow for allogeneic bone marrow transplantation. Leukaemic peripheral blood and marrow samples were similarly obtained from four B cell ALL patients. Both were collected in preservative free heparin, diluted in HBSS containing 2% fetal calf serum and 0·1% NaN3 (HFN), and the low density fraction ($d<1{\cdot}077$ g/cm^3) isolated by centrifugation on Ficoll–Hypaque.

Two-colour and three-colour immunorfluorescence staining

For two-colour indirect staining with anti-CD34, low-density cells (10^6/ml) were first incubated in 50 μl with a saturating concentration of MY10 or 8G12 MoAbs at 4°C for 30 min. Purified MY10 antibody was a kind gift from Dr C. I. Civin (Johns Hopkins Oncology Center, Baltimore, MD). After washing in HFN the cells were stained with FITC-conjugated F(ab′)$_2$ fragments of affinity purified sheep anti-mouse IgG antibodies (Cappel 1711–1744, Organon Teknika, West Chester, PA) for 30 min on ice. Cells were then incubated with an irrelevant mouse IgG1 to block free binding sites of the second step antibody. After washing, the cells were stained with PE-conjugated anti-CD19 (Leu12-PE, Becton Dickinson, Mountain View, CA) or anti-CD10 (J5-RD1; Coulter Immunology, Hialeah, FL).

For three-colour labelling experiments using MY10 and 8G12, the MY10 MoAb was added in the first step at 10 μg/ml final concentration. As a control, an irrelevant mouse IgG1 or a positively staining MoAb against a different antigen (anti-CD44R, 1A5; Dougherty, Lansdorp & Humphries, 1991) was used instead of MY10. After 30 min on ice, cells were washed twice in HFN and incubated with biotinylated goat anti-mouse IgG (Jackson Immuno-Research Laboratories, West Grove, PA) diluted 1/100 in HFN containing 10% normal human serum (HFN-NHS) for 30 min at 4°C, followed by washing and staining with streptavidin–Texas red–PE (SA-Duochrome, Becton Dickinson) diluted 1/20 in HFN-NHS containing 20 μg/ml of an irrelevant mouse IgG1 MoAb. After 10 min, excess (10 μg/ml) of 8G12-FITC, together with CD10-PE or CD19-PE was added for 20 min, prior to the final wash steps and analysis.

Immunofluorescence analysis was performed using a single argon ion (488 nm) laser FACStar Plus or FACScan machines (Becton Dickinson).

Cell sorting of CD10+ precursors

Low-density bone marrow cells were incubated at 10^7/ml in anti-CD10-PE with or without anti-CD16-FITC (Leu11b, Becton Dickinson) for 45 min at 4°C. Anti-CD16 reacts with Fcγ R-bearing large granular lymphocytes and neutrophils and was used to eliminate non-specific red immunofluorescence and CD10+ non-B cell lineage cells. After washing the cells, they were resuspended in HFN at 5×10^6/ml and sorted on FACStar Plus (Becton Dickinson). The cell sorter was calibrated prior to the experiments with 10 μm fluorescence microspheres. Compensation was set up for FITC and PE fluorescence using single stained samples.

Cells were sorted in the normal-C mode at 1500–2000 cells/sec and collected in HFN. CD10+ and CD10− fractions were sorted independently, stained with FITC-conjugated anti-CD2 (Leu5), CD19 (Leu12), CD16 (Leu11a), CD33 (MY9, Coulter Immunology) or CD34 (8G12), and then re-analysed using the same instrument settings.

RESULTS

Comparison of MY10 and 8G12 on normal bone marrow cells

Figures 1 and 2 show the results of a representative experiment in which low-density normal marrow cells were triple-stained with both 8G12 (FITC) and MY10 (Duochrome), and either anti-CD10-PE or anti-CD19-PE. Although 8G12 gives a stronger fluorescence signal than MY10 on triple stained bone marrow cells, all MY10+ marrow cells also bind 8G12 (Fig. 1c). Figure 1a, b shows the control profiles obtained with an irrelevant mIgG1, or a MoAb that does react with some human marrow cells (anti-CD44R) instead of MY10 to validate the triple-staining procedure.

Representative contour plots of 8G12 and MY10 versus CD10 or CD19 are shown in Fig. 2; panels a and b in this figure show the high degree of similarity in the general pattern of reactivity seen with both MoAbs. However, with 8G12 an association of the most intensely stained CD34+ cells with the CD10− and CD19− fractions was more clearly evident as shown by analysis of populations gated on CD34 using either 8G12 (panels c and d) or MY10 (panels e and f). Although CD34+ CD10−CD19− cells appear to express more CD34 when stained with 8G12 compared with MY10, the proportion of CD10+ or CD19+ cells in gates defined by either 8G12 or MY10 did not change.

Comparison of MY10 and 8G12 on ALL cells

In order to further compare directly the reactivity of MY10 and 8G12, peripheral blood and bone marrow cell samples from four ALL patients were indirectly stained with either (unlabelled)

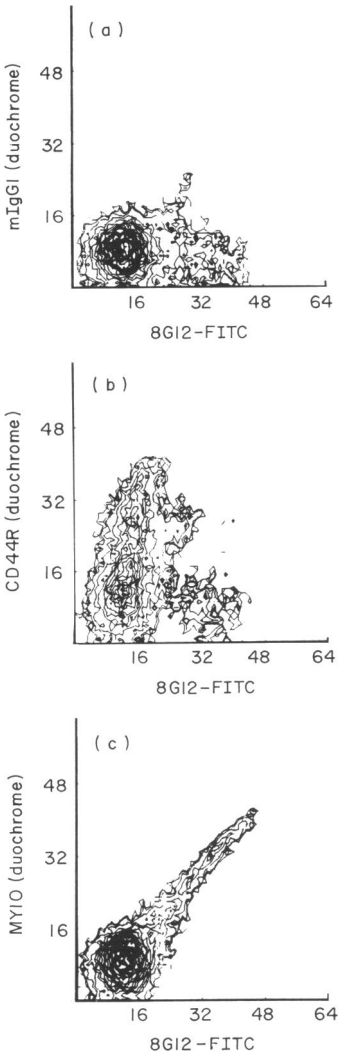

Fig. 1. Two-colour staining of low density normal bone marrow cells with 8G12-FITC and MY10 shows the same cells to be stained with both anti-CD34 MoAb. (a) Control in which cells were double stained with mIgG1; (b) control in which cells were double stained with anti-CD44R; (c) test in which cells were double stained with MY10. Fluorescence intensity is shown on a log scale.

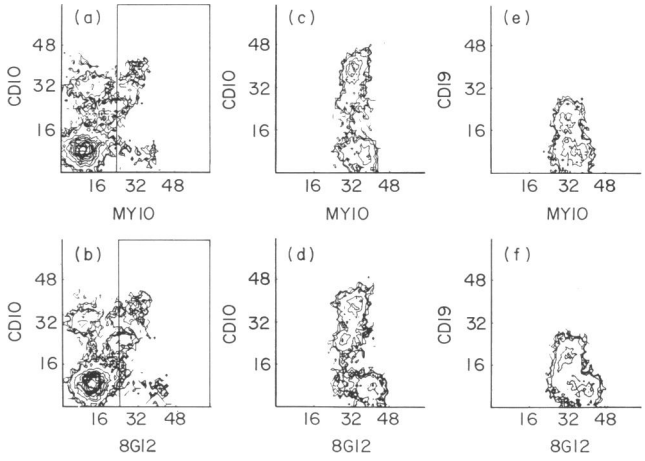

Fig. 2. Comparison of CD10 and CD19 expression on 8G12 *versus* MY10 labelled normal bone marrow cells assessed using three-colour flow cytometry. Low density bone marrow cells were stained with MY10 (duochrome) and 8G12-FITC, together with anti-CD10-PE (a–d) or anti-CD19-PE (e and f). To facilitate the comparison between 8G12 and My10, data collection was performed with gates set on cells positively stained with 8G12 (c and e) or MY10 (d and f) as defined by the rectangles shown in a and b, respectively.

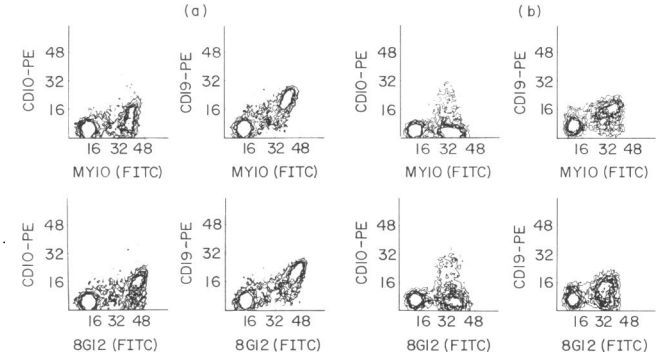

Fig. 3. Comparison of MY10 and 8G12 reactivity with bone marrow (a) and peripheral blood (b) cells from an ALL patient. Low density cells from bone marrow and peripheral blood were double stained with MY10 or 8G12 plus SAM-FITC, and anti-CD10-PE or anti-CD19-PE, after blocking of the remaining free binding sites of the sheep anti-mouse antibodies.

MoAb using anti-mouse immunoglobulin-FITC in combination with PE-labelled anti-CD10 or anti-CD19 and then two-colour immunofluorescence analysis was performed. The results of a representative experiment are depicted in Fig. 3. Both anti-CD34 MoAbs revealed the presence of significant populations showing high levels of CD34 expression and some CD10 and CD19 positivity on the majority of cells. Similar analyses of the cells from all four ALL patients studied confirmed in each case that both anti-CD34 MoAb reacted with the same populations with no detectable qualitative differences, although again 8G12 usually gave a higher overall fluorescence intensity.

CD34 subpopulation analyses

Having demonstrated that MY10 and 8G12 react with the same cell populations, we next used 8G12-FITC to investigate the phenotypes of early B cells progenitors in normal human marrow by examination of the expression of CD34 and CD19 among $CD10^+$ cells. The objective of this analysis was to determine whether a $CD34^+CD10^+CD19^-$ subpopulation could be detected. As shown in Fig. 4, when sorted $CD10^+$ cells were restrained with various FITC-conjugated MoAbs, the existence of three $CD10^+$ populations was indicated: a $CD10^+$ subset that co-expressed CD34 and CD19; a $CD10^+$ fraction (containing the majority of the sorted cells) that was almost all $CD34^-$ and $CD19^+$; and a $CD10^{low}$ $CD34^-CD19^-$ subpopulation. When the $CD10^{low}$ (and hence $CD34^-CD19^-$) cells were analysed with MoAbs specific for non-B-lineage markers, e.g. anti-CD16 and anti-CD33, positive labelling was found. Thus,

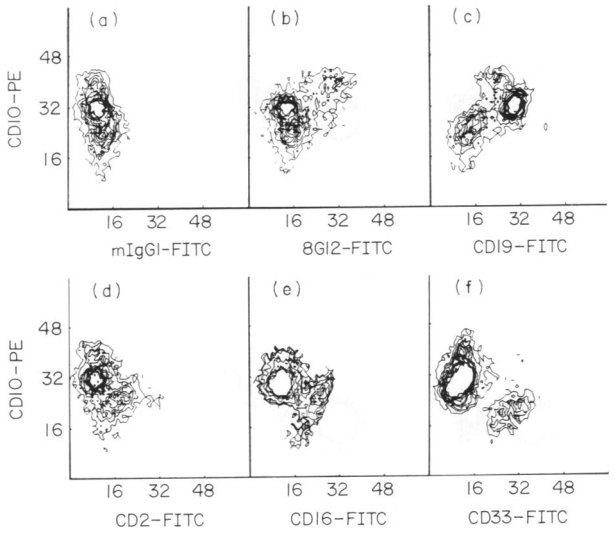

Fig. 4. Analysis of sorted CD10$^+$ bone marrow cells after relabelling with various FITC-conjugated MoAbs. Low density normal bone marrow cells were first stained with anti-CD10-PE and positive cells sorted. These were then restained with the indicated FITC-conjugated MoAbs, and re-analysed using the same instrument settings.

cells with a CD10^{low}CD34$^-$CD19$^-$ phenotype probably include cells from an early stage of granulopoiesis, since such cells are known to express some CD10, as well as large granular lymphocytes (NK cells) which express FcγR (CD16).

CD10$^+$CD19$^-$ subpopulation analyses

In a subsequent series of experiments, low density bone marrow cells were incubated with both anti-CD10-PE and anti-CD16-FITC and then sorted as depicted in Fig. 5. The data in this figure show that when the CD10$^+$CD16$^-$ population was restained with 8G12-FITC, the CD10^{bright} cells appeared to coexpress CD34 as indicated previously in Fig. 4. More importantly, after relabelling with anti-CD19-FITC, essentially all the cells from this CD10$^+$ sorted fraction were also found to express CD19 and no CD10$^+$CD19$^-$ cells were detected (at a sensitivity of $\leq 0{\cdot}1\%$) in either of two independent experiments. Figure 5 also shows the results of parallel relabelling of the CD10$^-$CD16$^-$ fraction, which revealed subpopulations of CD2$^+$ (T cells), CD19$^+$ (B cells) and a small number of CD34$^+$ (myeloid precursors) cells.

DISCUSSION

The restricted but consistent expression of CD34 on a variety of early human haematopoietic cells has focused attention on the potential of anti-CD34 MoAb-based strategies for isolating and characterizing specific progenitor populations (Lu *et al.*, 1987; Andrews *et al.*, 1989; Sutherland *et al.*, 1989; Thomas, Sutherland & Lansdorp, 1989; Lansdorp *et al.*, 1990). Several anti-CD34 MoAbs have now been described, most of which do not react with neuraminidase-treated KG1a cells and are unable to immunoprecipitate fully desialylated CD34 antigen (Sutherland *et al.*, 1988; Civin *et al.*, 1989). Variations in the glycosylation of CD34 molecules in different cell types might thus also affect the detection of specific subpopulations by MoAbs that are reactive with different epitopes on the CD34 molecule. Some heterogeneity might therefore be anticipated in comparisons of the binding patterns obtained when such CD34 MoAbs are used to stain normal and/or leukaemic haematopoietic cell populations. Discrepant reactivities of different anti-CD34 MoAbs with certain B-lineage ALL cells have been noted, although in these studies the alternative explanation, i.e. that the differences observed were due to differences in the titres of the MoAbs used, was not ruled out (Civin *et al.*, 1989). In light of these considerations, it was of interest to compare the cell binding patterns obtained with a new anti-CD34 MoAb we have recently isolated (8G12) and MY10, since these two antibodies have previously been shown to react with different epitopes (Lansdorp *et al.*, 1989). Our results of parallel staining of normal marrow, and leukaemic marrow and blood samples from four ALL patients described here, as well as several samples of regenerating marrow obtained from bone transplant recipients (data not shown) did not reveal any discordance in staining patterns that could not be attributed to a higher avidity of 8G12 suggested previously (Lansdorp *et al.*, 1989).

The ability of 8G12 to be coupled directly to a fluorochrome makes this an invaluable reagent for CD34$^+$ subpopulation analyses. A currently controversial area that can be approached using such a reagent, concerns the orderly sequence of phenotype changes that characterize human cells undergoing the earliest part of the B-lineage developmental program. Although multi-parameter flow cytometry has contributed significantly to defining distinct stages of early B cell progenitor development in humans, some discrepancies still exist regarding the order of expression of CD10 (CALLA) and CD19. Initially, data were sought from immunophenotype analyses of non-T cell ALL, because most leukaemic cells in such malignancies were thought to correspond to various normal cell types whose phenotype had been 'frozen' at the time of leukaemic transformation (Nadler *et al.*, 1984; Ryan *et al.*, 1987). Such studies showed that a small subset of ALL patients may possess leukaemic cells that lack surface immunoglobulin and CD10 but express CD19, suggesting that CD19 might be expressed before CD10 at an early stage during the development of pre-B cells. However, cells with a CD10$^-$CD19$^+$ phenotype, like cells with a CD10$^+$CD20$^+$ phenotype (also often observed in ALL), have not been found in normal human marrow or fetal liver (Uckun & Ledbetter, 1988; Loken *et al.*, 1987). Their demonstration in ALL might therefore now be interpreted as examples of aberrant or asynchronous marker expression (McCulloch, 1983; Anderson *et al.*, 1984; Hurwitz *et al.*, 1988; Smith & Kitchens, 1989).

More recent analysis of fetal liver and marrow has indicated that expression of CD10 may precede the expression of CD19 (Uckun & Ledbetter, 1988). However, because expression of CD10 is not exclusive to B-lineage cells (Letarte *et al.*, 1988), demonstration of terminal deoxynucleotidyl transferase production and the expression of other B-lineage properties is required to establish definitely the lymphoid nature of otherwise uncharacterized CD10$^+$ cells. Our results confirm this observation by revealing that some of the CD10^{low}CD34$^-$CD19$^-$ cells in adult marrow coexpress CD33 and CD16. However, existence of CD10$^+$CD19$^-$ early B cell precursors has been suggested in another recent study which showed that only 4–5% of normal circulating TdT$^+$, CD10$^+$ lymphocyte precursors express CD19 (Smith & Kitchens, 1989). However, attempts to date to identify a CD10$^+$CD19$^-$ population in adult or fetal bone marrow have not been successful (Loken *et al.*, 1987; LeBien *et al.*, 1990). The

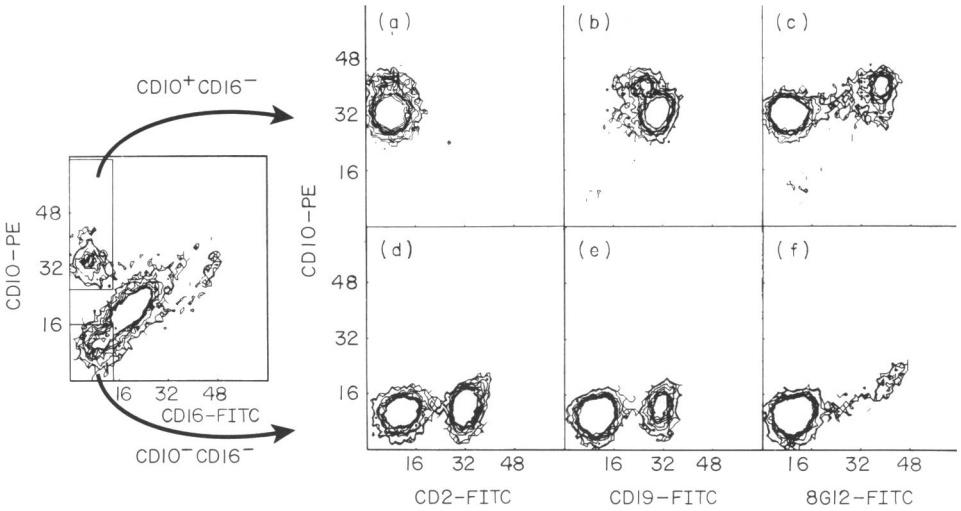

Fig. 5. Analysis of sorted CD16$^-$ normal bone marrow cells after relabelling with various FITC-conjugated MoAbs. Low density normal bone marrow cells were first double stained with anti-CD10-PE and anti-CD16-FITC, and then CD10$^+$CD16$^-$ and CD10$^-$CD16$^-$ fractions sorted as indicated. These two populations were then re-analysed after staining with FITC-conjugated MoAbs for CD2 (a and d), CD19 (b and e) or CD34 (8G12) (c and f) antigens using the same instrument settings.

sorting results described here also indicate that the vast majority of CD10$^+$ cells in adult normal marrow coexpress some CD19, although a significant proportion of these have a CD10^{bright}CD34$^+$CD19^{dull} phenotype. This phenotype might thus represent the earliest B-lineage cell phenotype identifiable in adult bone marrow. If these cells are being produced by CD34$^+$CD10$^+$CD19$^-$ precursors as has been reported in some studies of human fetal liver, the cells with this phenotype represent a very small subpopulation in adult marrow indeed. One possible explanation for the disparity between fetal liver and adult marrow may be the existence of slightly different sequences of alterations in the composition of the pre-B cell surface at different stages of ontogeny. In this regard it is interesting to note that two distinct developmental pathways for B cells in mice have recently been described (Herzenberg & Herzenberg, 1989). To address the possibility of variation in human B lineage developmental programs, further experiments in which the earliest stages of human pre-B cells can be followed and manipulated *in vitro* will be required.

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